

**COMPOSITIONS AND METHODS FOR PRODUCING VASCULAR OCCLUSION**

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The present invention relates generally to methods and compositions for targeting, delivering, and activating platelet-dependent vascular occlusion agents. In particular, antibodies carrying platelet binding agents are targeted to hyperplastic cells or tissues, such as the vasculature of solid tumor masses; the platelet binding agent then binds and activates platelets, which in turn bind and activate other platelets. This process results in the formation of a platelet-mediated thrombus-causing vessel occlusion.

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| <p>(54) Title: COMPOSITIONS AND METHODS FOR PRODUCING VASCULAR OCCLUSION</p> <p>(57) Abstract</p> <p>The present invention relates generally to methods and compositions for targeting, delivering, and activating platelet-dependent vascular occlusion agents. In particular, antibodies carrying platelet binding agents are targeted to hyperplastic cells or tissues, such as the vasculature of solid tumor masses; the platelet binding agent then binds and activates platelets, which in turn bind and activate other platelets. This process results in the formation of a platelet-mediated thrombus-causing vessel occlusion.</p>                                                                              |                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |

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## 1 (A) TITLE

**COMPOSITIONS AND METHODS FOR PRODUCING  
VASCULAR OCCLUSION**

## (B) CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not Applicable

## (C) FEDERAL SPONSORSHIP

Not Applicable

## (D) BACKGROUND OF THE INVENTION

## 9 (D1) FIELD OF THE INVENTION

The present invention is directed to compositions and methods for producing a therapeutic benefit by producing vascular occlusion using platelet activation as the initiating event. Compositions and methods of the invention involve targeting platelets to a site, such as a tumor site, using a binding agent, such as a bi-functional molecule, a portion of which binds to the site and another portion of which binds or immobilizes a platelet binding agent, such as circulating von Willebrand Factor (VWF).

## (D2) DESCRIPTION OF RELATED ART

17 Platelets function in the body to limit blood loss in the event of vascular damage. Normally, platelets circulate throughout the body with other cellular components of blood, bathed in a mixture of various plasma proteins, many of which play key roles in the clotting process. Upon exposure of vascular sub-endothelium, a complex series of events occurs to limit the loss of blood from the damaged vessel.

21 Platelets contacting components of the exposed sub-endothelium: 1) bind and adhere, 2) spread across the exposed surface, 3) activate as evidenced by release of granule contents, 4) aggregate and recruit other platelets from the blood stream, and 5) form an efficient

25 plug stemming the flow of blood from the vessel.

1 In contrast to the coagulation cascade, i.e., the sequential conversion of  
coagulation protein zymogens into active enzymes and which ultimately ends in the  
conversion of fibrinogen to fibrin, platelets bind specifically to the damaged area and are  
held together by bridging molecules that bind to specific receptors on the platelet  
5 surface. The initial bridging between platelets and the sub-endothelium is dependent on  
the interaction between the glycoprotein Ib (GPIb) receptor on the surface of the  
platelet and VWF in the subendothelium (i.e., immobilized VWF). This interaction in  
itself is unique since normal platelets circulating in the blood contacting soluble VWF  
9 are not activated, nor do they bind to the soluble VWF. In vitro experimentation has  
confirmed that immobilization of the soluble VWF to a surface facilitates binding and  
activation of platelets (Stewart et al, *British Journal of Haematology*, 97:231-9, 1997).  
Upon activation of the platelet an additional receptor, glycoprotein IIb/IIIa  
13 (GPIIb/IIIa), is altered and enables the binding of several plasma proteins, thereby  
promoting platelet/platelet binding (Savage et al, *Journal of Biological Chemistry*,  
267:11300-6, 1992). In addition to fibrinogen, soluble VWF binds to the activated  
GPIIb/IIIa receptor, in turn becoming immobilized and capable of binding other  
17 platelets via GPIb and GPIIb/IIIa.

Hyperactive platelets induce thrombus formation at inopportune times resulting  
in reduced blood supply to key organs and tissues. A prime example is thrombus  
formation induced by blood flowing through a stenotic (narrowed) vessel supplying the  
21 heart. Reduction of the flow of blood to the heart muscle leads to infarction and  
eventually heart attack (cardiac cell death). Cerebral ischemia (transient ischemic  
attack, TIA; stroke) occurs when an embolus or thrombus occludes blood vessels  
feeding the brain.

25 Other pathological states exist which are caused by platelet activation due to an  
antibody-mediated process. Heparin-induced thrombocytopenia (HIT) is characterized  
by a dramatic loss in platelet numbers and thrombus formation at sites of pre-existing  
pathology. Patients receiving heparin, as an anticoagulant to promote blood flow,  
29 occasionally (1 to 5% of all patients receiving un-fractionated heparin) produce an  
antibody that binds to heparin in complex with a platelet granule protein (Kelton et al,

1 *Blood*, 83:3232-9, 1994). The binding of the antibody to the heparin/protein complex  
on the surface of the platelet induces rapid platelet activation and localized thrombus  
formation. This in turn leads to infarction of the affected area.

Thrombosis is a well-described consequence of cancer. Controversy exists as to  
5 whether the presence of a hyper-coagulable state is predictive of cancer. Many studies  
have been conducted demonstrating a prothrombotic tendency with most neoplasias. It  
has been suggested that thrombosis is the most frequent complication in patients with  
overt malignant disease.

9 Concern has arisen regarding the potential risk of enhancing thromboembolic  
disease as a result of current therapy regimens (surgical or chemotherapeutic). In some  
instances, oral anticoagulation is initiated to prevent possible thrombotic complications.  
A key to the development of successful anti-tumor agents is the ability to design agents  
13 that will selectively kill tumor cells, while exerting relatively little, if any, untoward  
effects against normal tissues. This goal has been elusive to achieve in that there are few  
qualitative differences between neoplastic and normal tissues. Because of this much  
research over the years has focused on identifying tumor-specific "marker antigens" that  
17 can serve as immunological targets both for chemotherapy and diagnosis. Many tumor-  
specific or quasi-tumor-specific (tumor-associated) markers have been identified as  
tumor cell antigens that can be recognized by specific antibodies.

Unfortunately, it is generally the case that tumor specific antibodies will not in  
21 and of themselves exert sufficient anti-tumor effects to make them useful in cancer  
therapy. In contrast with their efficacy in lymphomas, immunotoxins have proven to  
be relatively ineffective in the treatment of solid tumors such as carcinomas. The  
principal reason for this is that solid tumors are generally impermeable to antibody-  
25 sized molecules: specific uptake values of less than 0.001% of the injected dose/g of  
tumor are not uncommon in human studies. Furthermore, antibodies that enter the  
tumor mass do not distribute evenly for several reasons. First, the dense packing of  
tumor cells and fibrous tumor stromas present a formidable physical barrier to macro-  
29 molecular transport and combined with the absence of lymphatic drainage create an  
elevated interstitial pressure in the tumor core which reduces extravasation and fluid

1 convection. Second, the distribution of blood vessels in most tumors is disorganized  
and heterogeneous; as a result, some tumor cells are separated from extravasating  
antibody by large diffusion distances. Third, all of the antibody entering the tumor  
may become absorbed in perivascular regions by the first tumor cells encountered,  
5 leaving none to reach tumor cells at more distant sites.

One approach would be to target cytotoxic agents or thrombus-inducing agents  
to the vasculature of the tumor rather than to the tumor.

The present inventors propose that this approach offers several advantages over  
9 direct targeting of tumor cells. First, the target cells are directly accessible to  
intravenously administered therapeutic agents permitting rapid localization of a high  
percentage of the injected dose. Second, since each capillary provides oxygen and  
nutrients for thousands of cells in its surrounding 'cord' of tumor, even limited damage  
13 to the tumor vasculature could produce an avalanche of tumor cell death. Finally, the  
outgrowth of mutant endothelial cells lacking the target antigen is unlikely because they  
are normal cells.

#### (E) SUMMARY OF THE INVENTION

17 The present invention relates to therapeutic methods and compositions for  
targeting tissues and/or organs, and associated vasculature, which are hyperplastic or  
neoplastic in nature, using agents that induce thrombus formation via localized platelet  
activation. The composition uses an agent for capturing platelets at a pre-determined  
21 site and an agent for activating the captured platelets. In some embodiments of the  
invention, a single agent both captures and activates the platelets. The method utilizes  
localized platelet collection and activation, which produces subsequent thrombus  
formation, thereby limiting the blood supply to the target area, without inducing a  
25 generalized or systemic pro-thrombotic state.

The invention also may employ bifunctional agents having a targeting  
component capable of binding tumor-associated antigens and/or antigens expressed on  
tumor vasculature, and a platelet-specific recognition component capable of binding and  
29 capturing platelets.

1 Purposeful induction of thrombosis in a cancer patient appears, at first glance, to  
be counter-intuitive, since inducing thrombosis in a cancer patient is well known to  
contribute significantly to patient morbidity and mortality.

5 The present invention, targeted platelet-mediated occlusion, is based on the site-  
specific induction of thrombosis, utilizing the body's natural capacity to produce a  
thrombus in response to immobilized von Willebrand factor (VWF) or other locally  
acting platelet activation agents. Although VWF circulates in the blood stream in  
soluble form, it is not until the molecule is exposed as part of the subendothelium or  
9 binds to exposed collagen from the subendothelium that it is capable of capturing  
platelets and inducing platelet activation.

Using a bifunctional targeting agent directed toward a pre-determined site or  
molecule (e.g. a tumor-specific or tumor-associated antigen, ligand/receptor complex),  
13 VWF is immobilized at the site, thereby inducing localized platelet capture and  
activation leading to thrombosis and cessation of blood flow to the local area. Cells,  
including tumor cells, diminish or die as a result of loss of localized blood flow. This  
approach avoids systemic platelet activation and thrombosis; relying on the fact that  
17 immobilized VWF (not soluble VWF) will capture and activate a circulating platelet.  
Thus, the methods and compositions of the present invention are an indirect means of  
treating a pathological condition, such as cancer or hyperplastic cells.

In a manner similar to an existing pathological condition (i.e. Heparin-Induced  
21 Thrombocytopenia, HIT), localized platelet activation can be enhanced via an Fc-  
mediated process by incorporating a human Fc fragment into the bifunctional anti-  
tumor-associated or tumor-specific antigen or ligand/receptor complex targeting agent.  
Platelet activation in HIT syndrome results in localized thrombosis and cessation of  
25 blood flow to the affected area. This leads to death of the local tissue.

The extent and degree of site-specific thrombosis can be controlled in a variety  
of ways. Inhibition of platelet activation through the use of anti-platelet agents (e.g.  
GPIIb/IIIa inhibitors, aspirin, dipyridamole, etc.) decreases the propensity to induce a  
29 thrombus, in a defined, titratable manner. Altering local blood flow, blood pressure  
and tissue temperature can also serve as means of controlling local platelet activation to



1 a stimulus.

Typical vascularized tumors are the solid tumors, particularly carcinomas, which require a vascular component for the provision of oxygen and nutrients. Exemplary solid tumors to which the present invention is directed, include, but are not limited to, carcinomas of the lung, breast, ovary, stomach, pancreas, larynx, esophagus, testes, liver, parotid, biliary tract, colon, rectum, cervix, uterus, endometrium, kidney, bladder, prostate, thyroid, squamous cell carcinomas, adenocarcinomas, small cell carcinomas, melanomas, gliomas, neuroblastomas, and the like.

9 A preferred method of the invention includes preparing a binding agent, such as an antibody or antibody-like molecule, that recognizes an antigen or other ligand associated with the vascular endothelial cells of the vascularized tumor mass; linking or operatively attaching the antibody to the selected agents to form an antibody-agent conjugate and introducing the antibody-agent conjugate into the bloodstream of an animal, such as a human patient or a test animal. As used herein however, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgE, F(ab')<sub>2</sub>, a univalent fragment such as Fab', Fab, Dab, as well as engineered antibodies such as single chain antibodies, covalently-linked single chain antibodies, recombinant antibodies, humanized antibodies, bispecific antibodies, and the like.

As noted above, a solution to the problem of poor penetration of antibodies into solid tumors is to attack the endothelial cells (EC) lining the blood vessels in the tumor. This approach offers several advantages over direct targeting of tumor cells. First, the target cells are directly accessible to intravenously administered therapeutic agents permitting rapid localization of high percentage of the injected dose. Second, since each capillary provides oxygen and nutrients for thousands of cells in its surrounding 'cord' of tumor even limited damage to the tumor vasculature could produce extensive tumor cell death. Finally, endothelial cells are similar in different tumors, making it feasible to develop a single reagent for treating numerous types of cancer.

29 An example of one such reagent would be that which recognizes a complex of VEGF and its receptor. Such an agent would be fashioned to uniquely recognize the

- 1 combination of VEGF and the VEGF receptor, having little or no effect on either the  
ligand (VEGF) or the receptor.

(F) DESCRIPTION OF THE DRAWINGS

Not Applicable

5 (G) DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods for capturing  
platelets at a pre-determined site, activating the platelets, and harnessing the natural  
function of platelets to achieve a beneficial therapeutic result. In accordance with the  
9 present invention, platelets may be targeted to a specific site, then the natural ability of  
platelets to induce thrombus formation may be used to interrupt, disrupt, or reduce  
blood flow at the site. Reduced blood flow concomitantly reduces nutrient supply to a  
disease or condition agent, such as a tumor, so the size of the disease agent is  
13 diminished. It is clear that reducing the size of a tumor is an obvious therapeutic  
benefit.

The present invention also provides compositions and methods for indirectly  
treating a disease or condition by disrupting blood flow to a site or locus of the disease.  
17 In this embodiment of the invention. Blood flow is disrupted by positioning platelets at  
a pre-determined site near but not on the site of the disease; activating the platelets; and  
allowing the natural function of the platelets, e.g., thrombus formation, to reduce  
and/or eliminate the nutrient supply to the disease site.

21 The present invention also includes targeting platelets to a pre-determined tissue  
capable of being selectively targeted, e.g., hyperplastic tissue, and activating the platelets.  
Activating the platelets at the pre-selected site causes a therapeutic benefit by reducing  
the nutrient supply to the tissue or site.

25 The present invention provides compositions and methods for inducing  
thrombus formation by capturing platelets at a selected site, inducing activation of the  
platelets, and allowing a thrombus to form. By capturing platelets on tumor endothelial  
cells (an example of a selected site), the compositions and methods of the present

1 invention may be used to treat cancer. Furthermore, the compositions and methods of  
the present invention provide a therapeutic benefit to the recipient of the composition.

The present invention also provides compositions and methods for treating  
cancer by inducing platelets to collect at a pre-determined site, and activating the  
5 platelets, thereby forming a therapeutically beneficial thrombus.

The present invention also provides compositions that include a binding agent  
having a first binding component and a second binding component, said first binding  
component targeting the binding agent to the pre-determined site, e.g., comprising a  
9 binding region for binding the binding agent to the pre-determined site; said second  
binding component comprising a binding region for binding platelets. Such  
compositions include bifunctional binding agents having an antigenic determinant and a  
platelet binding site.

13 Compositions according to the present invention may also include a ligand as a  
targeting agent, and an anti-ligand for binding the ligand and binding platelets.

Compositions according to the invention may also include one or more of the  
following: one or more platelet binding modulators (e.g., inhibitors or enhancers), one  
17 or more thrombus formation controllers or modulators, or one or more complement  
cascade components.

Methods according to the invention may also include one or more of the  
following: administering a binding agent capable of capturing platelets at a pre-  
21 determined site; inducing activation of the captured platelets; administering a  
bifunctional binding agent having an antigenic determinant and a platelet binding site;  
controlling thrombus generation by altering the temperature of one or more  
compositions of the invention; and/or altering the temperature at the pre-selected site.

25 Methods according to the invention may further include one or more of the  
following: administering one or more platelet binding modulators, administering one or  
more thrombus formation modulators, or administering one or more complement  
cascade components.

29 The present invention also includes a kit including a binding agent for targeting  
a pre-determined site and at least one of the following: a binding agent for binding

1 platelets; a ligand for binding the binding agent; a ligand conjugate; an anti-ligand for  
binding the ligand or the ligand conjugate; a platelet binding modulator (enhancer  
and/or inhibitor); a thrombus formation modulator; a complement cascade component;  
a complement cascade component inducer; and a binding agent for binding platelets that  
5 includes an anti-ligand. The kit may include a bifunctional binding agent, and/or a  
binding agent-ligand conjugate, and/or a platelet-binding agent -anti-ligand conjugate.

The compositions and methods of the present invention include any mechanism  
of delivering a composition to the pre-selected site, including but not limited to  
9 systemically, locally, orally, or topically.

In accordance with some embodiments of the invention, binding agents are used  
to capture platelets at a predetermined site.

#### Definitions:

13 As used herein, a binding agent or targeting moiety refers to any chemical or  
biological molecule for binding one substance to another. Typically the binding agent  
binds a defined population of cells, typically hyperplastic tissue, or a cancer cell. A  
molecule's function as a binding agent should not be limited by the structural  
17 mechanism of attachment. For example, a binding agent may bind a receptor, an  
antigenic determinant or epitope, an enzymatic substrate, or other biological structure  
linking the binding agent to a target cell or cell population. The binding agent may be a  
conjugate, and includes but is not limited to immunological conjugates, chemical  
21 conjugates (covalent or non-covalent), fusion proteins, and the like.

As used herein, a ligand-binding agent refers to a complementary set of  
molecules that demonstrate specific binding for each other. Preferably, a ligand binding  
agent is a targeting and a platelet binding agent. A ligand/anti-ligand pair generally  
25 binds with relatively high affinity, and for this reason, may be highly desirable for use  
with the present invention. A very well known ligand/anti-ligand pair is biotin and  
avidin. As used herein, avidin refers to avidin, streptavidin, neutravidin, derivatives and  
analogs thereof, and functional equivalents thereof. Avidin may bind biotin in a  
29 multivalent or univalent manner. Other exemplary ligand/anti-ligand pairs include, but

1 are not limited to, homophyllic peptides, "leucine zippers", zinc finger proteins/ds  
DNA fragment, enzyme/inhibitor, hapten/antibody, ligand/receptor, growth  
factor/growth factor receptor.

As used herein, a selected site, a pre-determined sited, targeting, and pre-targeting  
5 all refer to the location where the accumulation of platelets will provide a  
therapeutically beneficial result. Typically this involves target site localization of a  
targeting moiety. Such sites include but are not limited to the vasculature of solid  
tumors, tumor associated antigens, tumor specific antigens, hyperplastic tissues, and/or  
9 the extracellular matrix or subendothelium that is adjacent to or comprises any of these  
tissues or cells. The binding agent may be directed against any antigen of clinical  
significance, but preferably is directed against a tumor-specific or tumor-associated  
antigen.

13 As used herein, thrombus refers to any semi-solid aggregate of blood cells  
enmeshed in fibrin and clumps of platelets. In accordance with the invention, a  
thrombus is formed as a direct result of activated platelet accumulation at the pre-  
determined site. Thrombosis refers to the formation of a thrombus, typically within a  
17 blood vessel. Thrombogenic refers to substances that tend to cause thrombosis, or  
thrombus-forming.

As used herein, therapeutically beneficial, providing a therapeutic benefit or the  
like refers to a desirable change in the physiology of the recipient animal. In a preferred  
21 embodiment of the invention, the change is detectable. In accordance with the  
invention, any biological mechanism that involves activated platelets or platelet  
modulation may be used or harnessed to achieve a beneficial therapeutic result.  
Exemplary therapeutic benefits produced in accordance with the present invention  
25 include but are not limited to forming a thrombus, forming a platelet-mediated  
occlusion, eliminating a hyperplastic tissue or cells, eliminating a tumor and/or tumor  
cells, diminishing the size of a hyperplastic tissue, diminishing the size of a tumor,  
causing the hyperplastic tissue or tumor to become susceptible to additional therapies  
29 such as chemotherapy and/or radiation therapy or the like, and starving or reducing the  
nutrient supply to a hyperplastic tissue or cancer.

1 As used herein, "administering" refers to any action that results in exposing or  
contacting a composition containing a binding agent with a pre-determined cell, cells, or  
tissue, typically mammalian. Administering may be conducted *in vivo*, *in vitro*, or *ex*  
2 *vivo*. For example, a composition may be administered by injection or through an  
5 endoscope or catheter. Administering also includes the direct application to cells of a  
composition according to the present invention. For example, during the course of  
surgery, tumor or hyperplastic cells may be exposed. In accordance with an  
embodiment of the invention, these exposed cells (or tumors) may be exposed directly  
9 to a composition of the present invention, e.g., by washing or irrigating the surgical site,  
and/or the cells.

As used herein, VEGF refers to all members of the Vascular Endothelial Growth  
Factor family. As used herein, VEGF receptor refers to all members of the Vascular  
13 Endothelial Growth Factor Receptor family, including but not limited to FLT1/  
VEGFR, FLK1/KDR/VEGFR2, and FLT4/VEGFR3. As used herein "causing a tissue  
or tumor to become susceptible to additional therapies" refers to inducing a condition  
of low nutrient and/or oxygen supply to the tissue or tumor, through the method of  
17 the present invention including, but not limited to, forming a thrombus in the tumor  
vasculature and/or causing a reduced blood supply to the tumor.

As noted above, the invention employs a binding agent. In accordance with the  
present invention, the binding agent may function as a targeting agent, a platelet-  
21 binding agent, a platelet-activating agent, and/or as a portion of a conjugate for  
targeting, platelet binding, or platelet activation. In a preferred embodiment of the  
invention, the binding agent is bifunctional, preferably functioning as a targeting agent  
and as a platelet-binding agent.

25 The preferred binding and/or targeting agent is an antibody or antibody-like  
molecule, preferably a monoclonal antibody. A preferred binding agent is an antibody  
that binds hyperplastic tissue or cells, including but not limited to a tumor, a tumor  
associated antigen (TAA), or a tumor specific antigen (TSA). In a preferred embodiment  
29 of the invention, the binding agent (i.e., antibody or antibody-like molecules) would  
bind to the VEGF/VEGF receptor complex. In a further preferred embodiment of the

1 invention, the antibody or antibody-like molecule binding would recognize a neo-  
epitope (cryptic or previously unavailable epitope) formed by the interaction of a ligand  
and its receptor (e.g., growth factor/growth factor receptor interaction). In a further  
preferred embodiment of the invention, the binding of the antibody or antibody-like  
5 molecules to the ligand/receptor complex (e.g., growth factor/growth factor receptor  
complex) would not affect the function of either the ligand (e.g., growth factor) or the  
receptor (e.g., growth factor receptor).

Exemplary binding agents include, but are not limited to: monoclonal  
9 antibodies; polyclonal antibodies; native or naked antibodies; chimeric monoclonal  
antibodies; humanized antibodies; genetically engineered monoclonal antibodies;  
fragments of antibodies, selected from the group consisting of F(ab)<sub>2</sub>, F(ab')<sub>2</sub>, Fab,  
F(ab'), Dab, Fv, sFv, Fc, and minimal recognition unit; single chains representing the  
13 reactive portion of monoclonal antibodies ("SC-Mab"); modified antibodies, e.g.  
activated or chemically-activated antibodies; tumor-binding peptides; a protein,  
including receptor proteins; peptide; polypeptide; glycoprotein; lipoprotein, or the like,  
e.g., growth factors; lymphokines and cytokines; enzymes, immune modulators;  
17 hormones, for example, somatostatin; a ligand (with its complementary anti-ligand  
pair); oligonucleotides; any of the above joined to a molecule that mediates an effector  
function; and mimics or fragments of any of the above. Analogs of the above-listed  
targeting moieties that retain the capacity to bind to a defined target cell population  
21 may also be used within the claimed invention. In addition synthetic targeting moieties  
may be designed.

Exemplary binding agents are those that bind to at least one epitope on an  
antigen or the like disclosed in Nustad, et al, *Tumor Biology*, 17:196-219 (1996) and Nap,  
25 et al, *Tumor Biology*, 17:325-331 (1996); *Tumor Biology*, 19:390-420 (1998); and *Tumor  
Biology*, 19:21-29 (1998).

Monoclonal antibodies useful in the practice of the present invention include  
whole antibody and fragments thereof. Such monoclonal antibodies and fragments are  
29 producible in accordance with conventional techniques, such as hybridoma synthesis,  
recombinant DNA techniques and protein synthesis. Useful monoclonal antibodies

1 and fragments may be derived from any species (including humans) or may be formed  
as chimeric proteins, which employ sequences from more than one species. These  
include but are not limited to the hybridoma technique originally described by Kohler  
and Milstein [*Nature*, 256:495-497 (1975)]; the human B-cell hybridoma technique  
5 [Kozbor, et al., *Immunology Today*, 4:72 (1983)]; and the EBV transformation  
technique [Cole, et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc.,  
pp. 77-96 (1985)].

Exemplary proteins useful in the practice of this invention include but are not  
9 limited to proteins corresponding to known cell surface receptors (including low  
density lipoproteins, transferrin and insulin), fibrinolytic enzymes, anti-HER2, platelet  
binding proteins such as annexins, and biological response modifiers (including  
interleukin, interferon, erythropoietin and colony-stimulating factor).

13 Oligonucleotides, e.g., anti-sense oligonucleotides that are complementary to portions  
of target cell nucleic acids (DNA or RNA), are also useful as targeting moieties in the  
practice of the present invention. Oligonucleotides binding to cell surfaces are also  
useful.

17 Any growth factor may be used for such a targeting purpose so long as it binds  
to a tumor or tumor-associated endothelial cell. Suitable growth factors for targeting  
include but are not limited to VEGF/VPF (vascular endothelial growth factor/vascular  
permeability factor), FGF (which, as used herein refers to the fibroblast growth factor  
21 family of proteins), TGFb (transforming growth factor b), EGF and pleiotropin.  
Preferably the growth factor receptor to which the targeting factor binds should be  
present at a higher concentration on the surface of tumor-associated endothelial cells  
than on non-tumor associated endothelial cells. Most preferably, the growth factor  
25 receptor to which the targeting growth factor binds should further be present at a  
higher concentration on the surface of tumor-associated endothelial cell than on any  
non-tumor-associated cell type.

Functional equivalents of the aforementioned molecules are also useful as  
29 targeting moieties of the present invention. One targeting moiety functional equivalent  
is a "mimetic" compound, an organic chemical construct designed to mimic the proper



1 configuration and/or orientation for targeting moiety-target cell binding. Another  
targeting moiety functional equivalent is a short polypeptide designated as a "minimal"  
polypeptide, constructed using computer-assisted molecular modeling and mutants  
having altered binding affinity, which minimal polypeptides exhibit the binding affinity  
5 of the targeting moiety.

The Fv fragments of immunoglobulins have many significant advantages over  
whole immunoglobulins for the purpose of targeted tumor therapy, including better  
lesion penetration on solid tumor tissue and more rapid blood clearance, as well as  
9 potentially lower Fc-mediated immunogenicity. An exemplary single-chain Fv (scFv)  
binding agent may be engineered from the genes isolated from the variable regions of a  
tumor antibody. For example, a scFv binding agent may comprise a genetically  
engineered recombinant fusion protein that comprises a heavy chain (Vh) and a light  
13 chain (Vl) variable domain connected by an artificial linker and an effector domain.

An embodiment of the invention involves a targeting agent having a binding  
affinity for a marker found, expressed, accessible to binding, or otherwise localized on  
the cell surfaces of tumor-associated vascular endothelial cells as compared to normal  
17 non-tumor-associated vasculature. Further, certain markers for which a targeting agent  
has a binding affinity may be associated with the tumor-associated vasculature rather  
than on the tumor-associated endothelial cells, themselves. For example, such markers  
may be located on basement membranes or tumor-associated connective tissue.

21 In preferred embodiments of the invention, it will be desirable to prepare and  
employ an antibody having a relatively high degree of tumor vasculature selectivity,  
which might be expressed as having little or no reactivity with the cell surface of  
normal endothelial cells as assessed by immunostaining of tissue sections. Bi-specific  
25 antibodies useful in the practice of this aspect of the invention, therefore, will have a  
dual specificity recognizing a selected tumor cell surface antigen on the one hand, and  
on the other hand, recognizing a selected platelet specific agent.

Any composition that includes a binding and/or targeting agent according to the  
29 invention may be used to initiate in vivo therapeutic benefit, thrombus formation,  
and/or cell killing or diminution. The composition may include one or more

1 adjuvants, one or more carriers, one or more excipients, one or more stabilizers, one or  
more permeating agents (e.g., agents that modulated movement across a cell membrane),  
one or more imaging reagents, one or more effectors; and/or physiologically acceptable  
5 saline. Generally, adjuvants are substances mixed with an immunogen in order to elicit  
a more marked immune response. The composition may also include pharmaceutically  
acceptable carriers. Pharmaceutically acceptable carriers include but are not limited to  
saline, sterile water, phosphate buffered saline, and the like. Other buffering agents,  
dispersing agents, and inert non-toxic substances suitable for delivery to a patient may  
9 be included in the compositions of the present invention. The compositions may be  
solutions suitable for administration, and are typically sterile and free of undesirable  
particulate matter. The compositions may be sterilized by conventional sterilization  
techniques.

13 In a preferred embodiment of the invention, a suitable composition includes a  
binding agent that binds to an antigen. Typically, the tumor antigen recognized by the  
bi-specific antibodies employed in the practice of the present invention will be one that  
is located on the cell surfaces of the tumor being targeted. A large number of solid  
17 tumor-associated antigens have now been described in the scientific literature and the  
preparation and use of antibodies that bind these antigens are well within the skill of the  
art; of course, the tumor antigen that is ultimately selected will depend on the particular  
tumor to be targeted.

21 Exemplary antigens useful as targets in accordance with the present invention  
include, but are not limited to antigens associated with cancer including but is not  
limited to lung, colon, rectum, breast, ovary, prostate gland, head, neck, bone, immune  
system, or any other anatomical location. Exemplary antigens and/or pre-determined  
25 sites include but are not limited to GM2, Tn, sTn, Thompson-Friedenreich antigen,  
Glogo H, Le(y), MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, DEA,  
hCGbeta, HER2/neu, HER2, PSA, PSMA, KSA, CA 72-4, CA 19-9, CA 15-3, CA 125,  
TAG-72. The subject may be a human or animal subject. Other illustrative tumors and  
29 tumor markers are listed in U.S. Patents 5,075,218 and 5,776,427.

As noted above, a composition or method of the present invention includes a

1 platelet specific agent or component. Exemplary platelet specific agents or components  
include but are not limited to von Willebrand factor (VWF), osteopontin, fibrinogen,  
fibrin, fibronectin, vitronectin, collagen, thrombospondin, laminin, heparin, heparan  
5 sulfate, chondroitin sulfate, phospholipase A2, matrix metalloproteinases (MMPs),  
thrombin, glass, sialyl-lewis X, fibulin-1, platelet-endothelial cell adhesion molecule  
(PECAM), intercellular adhesion molecule 1 (ICAM-1), ICAM-2, MAC-1, LFA-1,  
PSGL-1, either singly or in combination.

As noted above, a composition or method of the present invention may include  
9 a platelet-mediated occlusion enhancer. The platelet-mediated occlusion enhancer may  
be a moiety that forms a portion of a bi-functional molecule as noted above, may be an  
ingredient in a composition according to the invention, and/or may be administered  
separately from a composition according to the invention. Those skilled in the art will  
13 recognize that it may be desirable to include or use a platelet-mediated occlusion  
enhancer when the individual receiving the therapy is in a state of compromised  
haemostasis. Under such conditions, the individual receiving the therapy has a  
propensity to bleed due to a pathological process that may have been acquired or is  
17 congenital in nature. Since the utility of the present invention is reliant upon the  
formation of a thrombus in the tissue or tumor vasculature after targeting platelets to  
the area, use of methods that augment platelet activation and/or the coagulation process  
could compensate for the individuals hemorrhagic tendencies. Examples of such  
21 conditions include, but are not limited to, haemophilia, von Willebrand's disease,  
coagulation factor deficiencies, Glanzmann's thrombasthenia, and Bernard Soulier  
Syndrome.

Exemplary platelet-mediated occlusion enhancers include but are not limited to  
25 ristocetin, thrombin, heparin-induced thrombocytopenia (HIT) antibodies or portions  
thereof, antiphospholipid antibodies (APA) or portions thereof, whole antibody  
molecules via an Fc-mediated mechanism, anti-LIBS antibodies, anti-CD9 antibodies,  
epinephrine, thrombin receptor activating peptide (TRAP), cathepsin G, elastase,  
29 arachidonate, platelet activating factor (PAF), thromboxane A2 (TxA2), TxA2  
mimetics, phospholipase A2 (PLA2), activators of protein kinase C (PKC), adenosine

1 diphosphate (ADP), inducers of cyclo-oxygenase 1 (COX-1), inducers of cyclo-  
oxygenase 2 (COX-2), collagen, VWF, matrix metalloproteinases (MMPs), heparin,  
heparan sulfate, chondroitin sulfate, ionophores, complement cascade components (e.g.,  
C5b-9) platelet microparticles, platelet membrane fractions.

5 As noted above, a composition or method of the present invention may include  
a platelet-mediated occlusion retarder or the like. The platelet-mediated occlusion  
retarder may be a moiety that forms a portion of a bi-functional molecule as noted  
above, may be an ingredient in a composition according to the invention, and/or may  
9 be administered separately from a composition according to the invention. Those  
skilled in the art will recognize that it may be desirable to include or use a platelet-  
mediated occlusion retarder when the individual receiving therapy based on the method  
of the present invention has an underlying propensity to thrombose (i.e. form clots too  
13 rapidly and/or in inappropriate locations in the body). Although the method of the  
present invention is directed to the formation of a thrombus in the tumor vasculature,  
individuals with a propensity to thrombose may form thrombi in inappropriate  
locations during the course of the therapy described by the present invention. Use of  
17 agents to reduce the rapidity and/or extent of thrombosis could be used to minimize  
the risk of forming thrombi in inappropriate locations in the body. Examples of  
conditions whereby the individual receiving therapy encompassed by the present  
invention may require the use of occlusion retarders are, but are not limited to,  
21 coronary artery disease, acute myocardial infarction, transient ischemic attack, stroke,  
high blood pressure, ATIII deficiency, Protein C deficiency, Protein S deficiency,  
heparin-induced thrombocytopenia, deep vein thrombosis, peripheral vascular disease  
and/or Factor V Leiden deficiency.

25 Exemplary platelet-mediated occlusion retarders include but are not limited to  
aspirin, aspirin-like compounds, nonsteroidal anti-inflammatory drugs (NSAIDS), nitric  
oxide releasing NSAIDS (NO-NSAIDS), ibuprofen, acetaminophen, ketoprofen,  
ticlopidine, clopidogrel, indomethacin, dipyridamole, omega-3 fatty acids, prostacyclin,  
29 nitric oxide, inducers of nitric oxide, inducers of nitric oxide synthase,  
proanthocyanidins, matrix metalloproteinase inhibitors (MMPIs, TIMPs), anti-

1 GPIIb/IIIa agents, anti- $\alpha$ v $\beta$ 3 agents, anti- $\alpha$ 2 $\beta$ 1 agents, anti-CD36 agents,  
aurintricarboxylic acid, thrombin receptor antagonists, thromboxane receptor  
antagonists, streptokinase, urokinase, tissue plasminogen activator (tPA).

5 In addition, it is known that platelets which have been cooled below their  
membrane phase transition temperature (i.e., < 15 degrees C) become irreversibly  
activated. Although the platelets function normally if transfused into a patient, the  
platelets are rapidly cleared from the body (i.e., approximately 24 hours, in contrast to  
normal circulating platelet life span of 7 to 10 days). Although these platelets are  
9 cleared rapidly, they bind with high avidity to immobilized VWF. Therefore,  
transfusion of cooled platelets provides an additional means to enhance thrombus  
formation at the target site. Therefore, one embodiment of the invention includes  
controlling platelet-mediated occlusion by administering platelets cooled as noted  
13 above.

As noted above, the targeting moiety may be, or may be bound to, one member  
of a binding pair. Methods according to the invention may require a time period  
sufficient for accumulation of the targeting moiety at the site of localization, for optimal  
17 target to non-target accumulation, for accumulation and binding of the second member  
of the binding pair, and/or for clearance of unbound substances.

In accordance with the invention, three or more step targeting or localization  
steps may be used. Many of these protocols are well known in the art (see, for example,  
21 U.S. patent 5,578,287 using a biotin/avidin protocol). Exemplary multiple step  
protocols include, but are not limited to, administering a binding agent-ligand,  
administering an anti-ligand to clear unbound binding agent and to localize bound  
binding agent-ligand, and administering an active agent-ligand. As used herein, active  
25 agent refers to any therapeutic agent that is active or becomes active and leads to a  
therapeutic benefit.

In accordance with a method of the invention, the binding agent must be capable  
of binding a pre-determined binding site or receptor, and may be administered to the  
29 patient by any immunologically suitable route. For example, the binding agent may be  
introduced into the patient by an intravenous, subcutaneous, intraperitoneal,

1 intrathecal, intravesical, intradermal, intramuscular, or intralymphatic route. The  
composition may be in solution, tablet, aerosol, or multi-phase formulation forms.  
Liposomes, long-circulating liposomes, immunoliposomes, biodegradable microspheres,  
micelles, or the like may also be used as a carrier, vehicle, or delivery system. Further  
5 more, using *ex vivo* procedures well known in the art, blood, plasma, serum, or cell  
components from the patient may be removed from the patient; optionally, it may be  
desirable to purify the antigen in the patient's blood; the blood, plasma, serum or  
cellular component may then be mixed with a composition that includes a binding  
9 agent according to the invention; and the treated blood, plasma, serum or cellular  
component is returned to the patient. The clinician may compare the responses  
associated with these different routes in determining the most effective route of  
administration. The invention should not be limited to any particular method of  
13 introducing the binding agent into the patient.

Administration may be once, more than once, and over a prolonged period. As  
the compositions of this invention may be used for patients in a serious disease state,  
i.e., life-threatening or potentially life-threatening, excesses of the binding agent may be  
17 administered if desirable. Actual methods and protocols for administering  
pharmaceutical compositions, including dilution techniques for injections of the present  
compositions, are well known or will be apparent to one skilled in the art. Some of  
these methods and protocols are described in Remington's Pharmaceutical Science,  
21 Mack Publishing Co. (1982).

A binding agent may be administered in combination with other binding agents,  
or may be administered in combination with other treatment protocols or agents, e.g.,  
chemotherapeutic agents.

25 As is well known in the art, a disadvantage associated with administering  
binding agents or binding agent conjugates *in vivo* includes non-target or undesirable  
target binding. It is therefore a desirable attribute of any administered composition to  
minimize non-target binding, to minimize non-target exposure to the binding agent or  
29 active agent, and/or to maximize clearance of non-bound binding agent, ligand, or  
active agent. Moreover, optimizing these attributes typically permits administering a

1 higher dose of active agent, a therapeutic agent, or an element of the process that  
activates a previously un-activated agent. Those skilled in the art are well versed in  
selecting the optimal parameters for administering the highest possible dose while  
remaining safely below the toxicity threshold.

5 In accordance with a preferred embodiment of the invention, therefore, un-  
activated platelets accumulate or are induced to accumulate at a pre-determined site,  
then the properly localized platelets are selectively activated.

The effectiveness of the present invention may be monitored by conventional  
9 assays that determine thrombus formation, morphametric studies of thrombus  
formation, tumor necrosis, tumor size, tumor morphology, and/or thrombus  
formation that results in tumor necrosis. One skilled in the art will recognize that  
other tests may be performed to assess or monitor therapeutic benefit.

13 Since some binding agents such as proteins are by themselves poor immunogens,  
their immunogenicity may be augmented by administration in immunological  
adjuvants and antigen delivery systems. The immunogenicity of a specific composition  
may also be increased or optimized by choice of delivery route. For example, the  
17 immunogenicity of compositions produced in accordance with the present invention  
that include a monoclonal antibody may be increased by choosing a mode of delivery  
that increases the direct contact between the binding agent and the antigen. The  
referred route is intravenous. Those skilled in the art are conversant with the various  
21 choices available, and why one route might be chosen over another route for a  
particular binding agent.

One skilled in the art will also recognize that liposomes, nanospheres, micelles,  
or microspheres may be used to administer a composition, and that such administration  
25 may result in a therapeutically desirable benefit.

It will be recognized by those skilled in the art that for certain congenital and  
pathological conditions, some of which are listed below, it is desirable to modify a  
composition or method of the present invention to compensate for a predisposition of  
29 the patient to bleed excessively or to thrombose. Under these circumstances, use of  
modifying agents, which either enhance or dampen a method or composition of the

1 invention, can be employed. The use of these modifying agents is predicted to  
minimize bleeding or clotting episodes. Moreover, the use of modifying agents enables  
controlled administration of a composition according to the invention under normal  
circumstances (i.e., normal hemostasis).

5 Exemplary conditions that may warrant using controllers, retarders, or agents  
that diminish a method or composition of the invention include but are not limited to  
pro-thrombotic or pro-coagulant conditions, such as Factor V<sup>Leiden</sup> deficiency,  
antiphospholipid syndrome (APS), Protein C and/or Protein S and/or Antithrombin  
9 III deficiency, deep vein thrombosis (DVT), pseudo-von Willebrands disease, Type IIb  
von Willebrands disease, peripheral vascular disease (PVD), and high blood pressure,  
among others. Exemplary conditions that may warrant using enhancers or agents that  
augment a method or composition of the invention include but are not limited to any  
13 condition that includes a risk of hemorrhage, including but not limited to coagulation  
factor deficiencies, hemophilia, thrombocytopenia, and anticoagulation therapy, among  
others. Also, controlling thrombus generation includes at least one of altering the  
temperature at the pre-determined site, altering the rate of blood flow at the pre-  
17 determined site, and altering the blood pressure at the pre-determined site.

For example, upon initiation of the vascular occlusion process, reversal or  
dampening of the associated prothrombotic condition may be necessary, as will be  
recognized by those skilled in the art. In such cases, administration of agents that  
21 reduce platelet reactivity will, in turn, reduce response to the vascular occlusion  
initiators. Such agents are readily known by those skilled in the art and include, but are  
not limited to: aspirin or aspirin-like compounds, NSAIDS, NO-NSAIDS, ibuprofen,  
acetaminophen, ketoprofen, ticlopidine, clopidogrel, indomethacin, omega-3 fatty acids,  
25 prostacyclin, nitric oxide, inducers of nitric oxide, inducers of nitric oxide synthase,  
proanthocyanidins, matrix metalloproteinase inhibitors (MMPs, TIMPs), anti-GPIb  
agents, anti-GPIIb/IIIa agents, anti- $\alpha$ v $\beta$ 3 agents, anti- $\alpha$ 2 $\beta$ 1 agents, anti-CD36 agents,  
aurintricarboxylic acid, thrombin receptor antagonists, thromboxane receptor  
29 antagonists, streptokinase, urokinase, tissue plasminogen activator (tPA).

An exemplary process in which it may be desirable to enhance or augment



1 platelet occlusion process includes thrombocytopenic (low platelet count) patients.  
These individuals would benefit from concomitant or pre-administration (transfusion)  
of platelet products to provide an adequate resource of platelets to accomplish platelet  
occlusion. It will be recognized by those skilled in the art that all transfusable products  
5 mimicking or approximating normal platelet function can be used under such  
circumstances. Such agents include but are not limited to: random donor platelets,  
apheresis platelets, autologous platelets, washed platelets, platelet membrane fractions,  
cooled platelets, frozen platelets, particles containing or expressing platelet membrane  
9 components and whole blood.

As a further example, specific platelet-function enhancing agents can be  
employed to boost or enhance initial platelet reactivity once targeted to the site of  
therapy. Agents known to those skilled in the art have been demonstrated to enhance  
13 existing platelet reactivity and/or lower the threshold limiting sufficient platelet  
reactivity to facilitate irreversible platelet adhesion and/or platelet degranulation and/or  
platelet/platelet binding and/or platelet accretion about an existing thrombus. These  
agents include but are not limited to: ristocetin, thrombin, heparin-induced  
17 thrombocytopenia (HIT) antibodies or portions thereof, antiphospholipid antibodies  
(APA) or portions thereof, whole antibody molecules via an Fc-mediated mechanism,  
anti-ligand-induced binding site (anti-LIBS) antibodies or portions thereof, anti-CD9  
antibodies or portions thereof, epinephrine, thrombin receptor activating peptide  
21 (TRAP), cathepsin G, elastase, arachidonate, thromboxane A2 (TxA2) mimetics, TxA2,  
phospholipase A2 (PLA2), activators of protein kinase C (PKC), adenosine diphosphate  
(ADP), collagen, VWF, matrix metalloproteinases (MMPs), heparin, heparan sulfate,  
chondroitin sulfate, ionophores, platelet microparticles, platelet membrane fractions.

25 An exemplary method of targeting or pre-targeting a composition according to  
the invention includes accumulating the targeting agent at the target site, then  
administering a rapidly clearing composition comprising a complementary agent that is  
capable of binding the previously localized binding agent. Some of these techniques are  
29 disclosed in U.S. Patent 4,863,713.

Once introduced into the bloodstream of an animal bearing a tumor, such a

- 1 bispecific construct will bind to tumor cells within the tumor; bind or immobilize a  
platelet specific component, whereby immobilization activates the platelet specific  
component; the activated platelet specific component then binds and activates platelets;  
and the activated platelets in turn bind and activate other platelets until an occlusion is  
5 formed.

## EXAMPLES

### Example 1.

- The technique of preparing monoclonal antibodies against antigenic cell surface  
9 markers is quite straightforward and may be readily carried out using techniques well  
known to those of skill in the art as exemplified by the technique of Kohler and  
Milstein (1975). Generally speaking, the preparation of monoclonal antibodies using  
stimulated endothelial cells involves the following procedures. Cells or cell lines  
13 derived from human tumors are grown in tissue culture for  $\geq 4$  days. The tissue culture  
supernatant ("tumor-conditioned medium") is removed from the tumor cell cultures  
and added to cultures of human umbilical vein endothelial cells (HUVEC) at a final  
concentration of 50% (v/v). After 2 days culture the HUVEC are harvested non-  
17 enzymatically and  $1-2 \times 10^6$  cells injected intraperitoneally into mice. This process is  
repeated three times at two-weekly intervals, the final immunization being by the  
intravenous route. Three days later the spleen cells are harvested and fused with SP2/0  
myeloma cells by standard protocols (Kohler and Milstein, 1975): Hybridomas  
21 producing antibodies with the appropriate reactivity are cloned by limiting dilution.

- From the resultant collection of hybridomas, one will then select one or more  
hybridomas that produce an antibody that recognizes the activated vascular  
endothelium to a greater extent than it recognizes non-activated vascular endothelium  
25 of course, the ultimate goal is the identification of antibodies having virtually no  
binding affinity for normal endothelium. Suitable antibody-producing hybridomas are  
identified by screening using e.g., an ELISA, RIA, IRMA, IEF, or similar immunoassay  
against one or more types of tumor-activated endothelial cells. Once candidates have  
29 been identified, one will desire to test for the absence of reactivity for non-activated or

- 1 "normal" endothelium or other normal tissue or cell type. In this manner, hybridomas  
producing antibodies having an undesirably high level of normal cross-reactivity for the  
particular application envisioned may be excluded.

Example 2.

- 5 The technique of preparing single chain antibodies that specifically recognize a  
ligand/receptor complex, specifically a growth factor/growth factor receptor complex is  
employed, whereby the resulting antibody molecules recognize the growth  
factor/growth factor receptor complex, but do not bind to either the growth factor or  
9 growth factor receptor alone. These antibodies can be formed through the  
immunization of mice with a complex of purified ligand and receptor, such as VEGF  
and VEGF receptor, and the resulting V genes used to construct an antibody library in  
filamentous phage. The phage display of antibody fragments allows the production of  
13 recombinant antibody molecules against activated endothelial cell antigens, specifically a  
ligand/receptor complex. The phage system mimics the vertebrate immune system.

- Female Balb/C mice are immunized with HPLC-purified recombinant VEGF  
and VEGF receptor (e.g. soluble FLT) in complex, in the presence of an adjuvant such  
17 as Quil A. After the appropriate antibody titre is reached (approximately the fourth  
boost), the mice are sacrificed and the spleens isolated. Messenger RNA (mRNA) is  
isolated from the spleen and transcribed to cDNA. The V genes of the cDNA are  
amplified and assembled as "single chain Fv" (scFv). After digestion with the  
21 appropriate restriction enzymes, the scFv are ligated into phagemid vectors.  
Competent E. coli cells are then transformed with these phagemid libraries, and after  
infection with helper phage (e.g. M13K07, Pharmacia), phage particles displaying the  
scFv are prepared. Selected clones are screened for expression of soluble scFv binding to  
25 the ligand/receptor complex, but do not bind to either the ligand alone or the receptor  
alone. This screening is accomplished using standard ELISA techniques, with the  
ligand/receptor complex, ligand and receptor used as solid-phase antigens, respectively.

1 Example 3.

A variety of endothelial cell markers are known that can be employed as inducible targets for the practice of this aspect of the invention including VEGF/VPF (vascular endothelial growth factor/vascular permeability factor), endothelial-leukocyte  
5 adhesion molecule (ELAM-1; Bevilacqua et al., 1987); vascular cell adhesion molecule-1 (VCAM-1; Dustin et al.; 1986) intercellular adhesion molecule-1 (ICAM-1; Osborn et al., 1989); the agent leukocyte adhesion molecule-1 (LAM-1 agent) or even a major histocompatibility complex (MHC) Class II antigen, such as HLA-DR, HLA-DP, or  
9 HLA-DQ (Collins et al., 1984). Of these, the targeting of the VEGF/VEGF receptor complex will be preferred.

Example 4.

Targeting platelets to a specific site can take the form of establishing a linkage  
13 between the bi-functional binding agent and the target site, followed by platelet binding; or can take the form of establishing a linkage between the bi-functional binding agent and the platelet, followed by binding of the platelet to the target site. The latter example requires pretreatment of the platelets (in vivo, in vitro; autologous donor) with  
17 the bifunctional agent. An exemplary bi-functional agent would impart minimal immunogenicity to the treated platelets and would bind specifically with high avidity to the tumor site, tumor vasculature, hyperplastic tissue(s) or organ(s). Platelets pre-treated with the bi-functional agent could take the form of random donor or apheresis  
21 platelets suitable for transfusion. Platelets, which have been cooled before or after treatment with the bi-functional agent, would provide a means of enhancing the targeted vaso-occlusive effects.

Example 5.

25 Platelet activation at the target site will induce secondary effects that may enhance diminution or killing of the target tissue. Release of agents by the activated platelets such as platelet factor 4 (PF4) will inhibit angiogenesis. Platelet release of chemoattractants such as Regulated on Activation Normal T-cell Expressed and

- 1 Secreted (RANTES), post activation, will enhance the effects of leukocytes (e.g., eosinophils, monocytes) on target tissue. Expression of granular constituents such as CD62 by the platelets, post activation, will induce binding of monocytes and polymorphonuclear leukocytes (PMNs) resulting in tissue factor expression (monocyte; 5 procoagulant) and cellular activation and attack (PMNs). In addition, release of CD40 ligand (CD40L) by activated platelets at the target site will induce tissue factor expression by monocytes leading to a local hyper-coagulable state.

9 **Example 6.** *Identification of Candidate Monoclonal Antibodies*

Prostate cancer tumor cell line LnCAP (Clone FGC, ATCC # CRL-1740) was grown in modified RPMI 1640 medium according to the product information sheet provided by the American Type Culture Collection (ATCC). Six different hybridomas 13 producing antibodies specific for MUC1 were tested for binding reactivity to LnCAP cells and MCF-7 cells (human breast carcinoma) by flow cytometry. The following table describes the binding epitope specificity of these hybridomas and the reactivity of the hybridomas with MUC1-expressing MCF-7 human breast carcinoma cells.

|    |                  |                             |                                                           |
|----|------------------|-----------------------------|-----------------------------------------------------------|
| 17 | <b>Hybridoma</b> | <b>MUC1 Binding Epitope</b> | <b>Binding to MCF-7 Cells Flow Cytometry (% positive)</b> |
|    | Control          | -                           | 2%                                                        |
|    | TH1              | GVTAPDTRPAP                 | 72%                                                       |
|    | TH2              | PDTRP                       | 69%                                                       |
| 21 | TH3              | TSAPDTR                     | 12%                                                       |
|    | TH4              | APDTR                       | 17%                                                       |
|    | TH5              | TSAPDTR                     | 11%                                                       |
|    | TH6              | SAPDTRPA                    | 60%                                                       |
| 25 | TH1-TH6 Cocktail | -                           | 55%                                                       |

A similar study was carried out using prostate cancer LnCAP cells as the target. Also included in this study was antibody 7E11 (ATCC # HB 10494) recognizing prostate specific membrane antigen (PSMA) and EMT6/Ed (murine mastocytoma) cells 29 as target. The following table presents data from these flow cytometry experiments.

| Antibody         | LnCAP | EMT6/Ed |
|------------------|-------|---------|
| Control          | 8%    | 8%      |
| 7E11             | 58%   | 4%      |
| TH1-TH6 Cocktail | 55%   | 9%      |

Based on these results, the TH6 hybridoma (anti-MUC1) and the 7E11 hybridoma (anti-PSMA) were chosen for further study.

**Example 7.** *Biotinylation of Targeting Antibodies*

Protein A Sepharose-purified TH6 and 7E11 monoclonal antibodies were biotinylated according to the manufacturer's (Sigma, St. Louis, MO) directions. In addition, humanized (i.e., human Fc bound to mouse monoclonal F(ab')<sub>2</sub>) antibodies (TH6, 7E11) were also biotinylated. In a similar manner, purified human von Willebrand factor was also biotinylated. Testing confirmed both significant levels of biotinylation of the antibodies and no reduction in antibody binding to LnCAP cells. Similarly, biotinylated VWF was shown to retain both the capacity to initiate platelet adhesion and promote platelet/platelet binding, post biotinylation. VWF ( $\pm$  biotinylation) was immobilized on polystyrene beads as described by Stewart et al (Br J Haematol, 97:321-329, 1997). The VWF-coated beads were then used as a solid-phase agonist to induce platelet adhesion and subsequent platelet dense granule ATP release. ATP release was quantified using a luciferin/luciferase assay (Stewart et al, 1997). Comparison of VWF beads with biotinylated-VWF beads indicated the two agents to be equivocal relative to platelet activation ( $0.41 \pm 0.05$ ,  $0.39 \pm 0.05$  arbitrary luminescence units; respectively).

**Example 8.** *Targeting Platelets to Cancer Cells*

LnCAP cells were challenged with biotinylated antibodies, or non-biotinylated antibodies (TH6 or 7E11); washed to remove unbound antibody; challenged with avidin or neutravidin, or not; washed to remove unbound avidin or neutravidin; challenged with biotinylated human VWF; then incubated with human citrated whole blood or platelet rich plasma under agitation. Platelet reactivity with the LnCAP cells was then

- 1 assessed by video microscopy, phase contrast microscopy and fluorescence microscopy.  
The following table presents the results of these experiments.

| Test Conditions                               | Human Platelets<br>Binding<br>to LnCAP Cells |
|-----------------------------------------------|----------------------------------------------|
| Control - No agents added to LnCAPs           | -                                            |
| 5 TH6.biotin / No Avidin / VWF.biotin         | -                                            |
| TH6.biotin / Avidin / VWF.biotin              | ++                                           |
| Humanized TH6.biotin / No Avidin / VWF.biotin | -/ +                                         |
| Humanized TH6.biotin / Avidin / VWF.biotin    | ++++                                         |
| 9 7E11.biotin / No Avidin / VWF.biotin        | -                                            |
| 7E11.biotin / Avidin / VWF.biotin             | +++                                          |
| Humanized 7E11.biotin/ No Avidin / VWF.biotin | -/ +                                         |
| Humanized 7E11.biotin/ Avidin / VWF.biotin    | ++++                                         |

- 13 Targeting of the LnCAP cells with either biotinylated TH6 or 7E11 led to  
localization of platelets, in either platelet rich plasma or whole blood, to the cancer  
cells, via interaction with cancer cell-immobilized VWF. The interaction of platelets  
with cancer cells was dramatically enhanced by constructing a humanized/biotinylated  
17 targeting antibody. Engagement of the platelet Fc receptor by the humanized portion  
of the targeting antibody (i.e., the human Fc component) caused massive platelet  
aggregation about the cancer cells and subsequent membrane blebbing and/or loss of  
cell membranes in the form of vesicles.

- 21 Although the present invention has been described in terms of particular  
preferred embodiments, it is not limited to those embodiments. Alternative  
embodiments, examples, and modifications, which would still be encompassed by the  
invention, may be made by those skilled in the art, particularly in light of the foregoing  
25 teachings.

## 1 (H) CLAIMS

We claim:

1. A composition for inducing thrombus formation comprising a binding agent having a first binding component and a second binding component, said first  
5 binding component comprising a binding region for binding the binding agent to a pre-determined site; said second binding component comprising a binding region for binding platelets.
2. The composition of claim 1 wherein the first binding component is an antigen  
9 binding site.
3. The composition of claim 2 wherein the first binding component is one or more binding agents selected from the group consisting of an antibody, a monoclonal antibody, a polyclonal antibody, a humanized monoclonal antibody, a chimeric  
13 antibody, a single chain antibody, a dimeric single chain antibody construct, a multimeric single chain antibody construct, a peptide, a nucleic acid sequence, a protein, a ligand, an oligonucleotide, conjugates that include any one of the above, fragments or parts of any of the above, and functional equivalents of any  
17 of the above.
4. The composition of claim 2 wherein the first binding component binds to a neo-epitope.
5. The composition of claim 1 wherein the second binding component includes at  
21 least one of the components selected from the group consisting of von Willebrand factor, osteopontin, fibrinogen, fibrin, fibronectin, vitronectin, collagen, thrombospondin, laminin, heparin, heparan sulfate, chondroitin sulfate, phospholipase A2, matrix metalloproteinases, thrombin, glass, sialyl-  
25 lewis X, fibulin-1, PECAM, ICAM-1, ICAM-2, p-selectin ligand, MAC-1, LFA-



- 1 1, portions of any of the above, and functional equivalents of any of the above.
6. The composition of claim 1 further comprising a platelet binding enhancer.
7. The composition of claim 6 wherein the platelet binding enhancer comprises at least one of ristocetin, platelet microparticles, and platelet membrane portions.
- 5 8. The composition of claim 1 further comprising a thrombus formation modulator.
9. The composition of claim 8 wherein the thrombus formation modulator is one or more enhancers selected from the group consisting of inhibitors of  
9 fibrinolysis, inhibitors of anti-coagulant proteins, and tissue factor pathway inhibitor.
10. The composition of claim 9 wherein the anti-coagulant proteins include at least one of protein C, protein S, and anti-thrombin III.
- 13 11. The composition of claim 9 wherein the inhibitors of fibrinolysis include plasminogen activator inhibitors.
12. A method of inducing thrombus in vivo comprising:  
capturing platelets at a selected site;  
17 inducing activation of the platelets; and  
allowing a thrombus to form.
13. The method of claim 12 wherein inducing platelets to collect at a pre-determined site comprises administering a targeting agent that specifically binds platelets.
- 21 14. The method of claim 13 wherein the targeting agent is one or more binding

- 1 factor/growth factor receptor.
21. The method of claim 20 wherein the growth factor/growth factor receptor is VEGF/VEGF receptor or a peptide mimetic of VEGF or VEGF-like molecule bound to the VEGF receptor.
- 5 22. A kit for inducing thrombus formation comprising a binding agent for targeting a pre-determined site and at least one of the following: a binding agent for binding platelets; a ligand for binding the binding agent; a ligand conjugate; an anti-ligand for binding the ligand or the ligand conjugate; a platelet binding  
9 enhancer; a thrombus formation modulator; a complement cascade component; a complement cascade component inducer; and a binding agent for binding platelets that includes an anti-ligand.
- 13 23. The kit of claim 22 wherein the binding agent for targeting a pre-determined site includes a binding component for binding platelets.
24. The kit of claim 22 wherein the binding agent for targeting a pre-determined site includes a ligand.

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 7 A61K47/48 A61K39/395 //(A61K39/395, 38:48), (A61K39/395, 38:36), (A61K39/395, 38:57)

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                            | Relevant to claim No. |
|------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X          | WO 96 01653 A (SCRIPPS RESEARCH INST<br>;THORPE PHILIP E (US); UNIV TEXAS (US);<br>EDGI) 25 January 1996 (1996-01-25)<br>page 5, line 36 -page 6, line 21<br>page 14, line 25-35<br>page 18, line 25 -page 19, line 19<br>page 96, line 24 -page 98, line 5                                   | 1-24                  |
| X          | THORPE P: "Antibody -directed targeting<br>of tumor vasculature (Meeting abstract)."<br>PROC ANNU MEET AM ASSOC CANCER RES,<br>(1996). VOL. 37, PP. 668. ISSN:<br>0197-016X., XP002132126<br>University of Texas Southwestern Medical<br>Center, Dallas, TX 75235-8593.<br>the whole document | 12,22,24              |
|            | -/-                                                                                                                                                                                                                                                                                           |                       |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

**\* Special categories of cited documents:**

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

Date of the actual completion of the international search

3 March 2000

Date of mailing of the international search report

17/03/2000

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                       | Relevant to claim No. |
|------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X          | <p>BARINAGA M: "Designing therapies that target tumor blood vessels<br/>'news;comment!."</p> <p>SCIENCE, (1997 JAN 24) 275 (5299) 482-4. ,<br/>XP002132127</p> <p>page 482, column 2, paragraph 2<br/>page 483, column 1, paragraph 3<br/>page 484, column 2, paragraph 4 -column 3,<br/>paragraph 2</p> | 12,22,24              |
| A          | <p>WO 98 31394 A (KING STEVEN W ;GAO BONING<br/>(US); THORPE PHILIP E (US); UNIV TEXAS ( )<br/>23 July 1998 (1998-07-23)<br/>page 4, line 1-18<br/>page 10, line 4-19</p>                                                                                                                                | 1-24                  |

# INTERNATIONAL SEARCH REPORT

national application No.

PCT/IB 99/01809

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark: Although claims 12-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based based on the alleged effects of the compound/composition.**
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/IB 99/01809

| Patent document<br>cited in search report |   | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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